Requirements for Peptide-induced T Cell Receptor Downregulation on Naive CD8+ T Cells

By Zeling Cai,[‡] Hidehiro Kishimoto,[*] Anders Brunmark,[‡] Michael R. Jackson,[‡] Per A. Peterson,[‡] and Jonathan Sprent[*]

From the [*]Department of Immunology, IMM4, The Scripps Research Institute, La Jolla, California 92037; and [‡]R.W. Johnson Pharmaceutical Research Institute, San Diego, California 92121

Summary

The requirements for inducing downregulation of α/β T cell receptor (TCR) molecules on naive major histocompatibility complex class I–restricted T cells was investigated with 2C TCR transgenic mice and defined peptides as antigen. Confirming previous results, activation of 2C T cells in response to specific peptides required CD8 expression on the responder cells and was heavily dependent upon costimulation provided by either B7-1 or ICAM-1 on antigen-presenting cells (APC). These stringent requirements did not apply to TCR downregulation. Thus, TCR downregulation seemed to depend solely on TCR/peptide/interaction and did not require either CD8 or B7-1 expression; ICAM-1 potentiated TCR downregulation, but only with limiting doses of peptides. TCR downregulation was most prominent with high affinity peptides and appeared to be neither obligatory nor sufficient for T cell activation. In marked contrast to T cell activation, TCR downregulation was resistant to various metabolic inhibitors. The biological significance of TCR downregulation is unclear, but could be a device for protecting T cells against excessive signaling.

Stimulation of T cells via the TCR/CD3 complex elicits a complex signaling cascade which leads to cell activation, proliferation, and differentiation into effector cells (1, 2). In certain situations, TCR ligation is associated with receptor downregulation. This process is easily seen with anti-TCR antibodies and reflects receptor endocytosis via clathrin-coated pits followed by degradation in lysozomes (3–12). TCR downregulation also occurs when T cells recognize specific antigen, i.e., peptides bound to MHC molecules expressed on APCs (3, 13, 14).

The biological significance of TCR downregulation is still unclear. Recently, Lanzavecchia et al. have provided impressive evidence that TCR downregulation is an essential feature of T cell activation (15, 16). These workers view rapid internalization of the TCR after contact with antigen on APCs as a device to enable a large number of TCR molecules to make contact with a limited number of antigenic epitopes on the APCs. This model of sequential interaction of a series of TCRs with individual antigenic epitopes follows from the authors' finding that T cell stimulation requires engagement of a considerable number of surface TCR molecules, i.e., ~8,000/cell; this number is reduced by about fivefold with APCs expressing B7 molecules (16). The precise connection between TCR downregulation and T cell activation is unclear. One possibility is that TCR internalization serves to focus TCR-associated kinases in the vicinity of downstream substrates, thereby promoting or facilitating intracellular signaling (17). An alternative explanation for TCR downregulation is that internalization of the receptors limits prolonged contact with antigen, and thereby reduces the possibility that the T cell is tolerized or destroyed through excessive TCR signaling. This notion raises the question of whether TCR downregulation is an invariable feature of T cell activation.

The existing data on TCR downregulation are based almost entirely on studies with T cell clones and/or with anti-TCR antibody as a surrogate antigen. Hence, there is a need to define the requirements for inducing TCR downregulation on naive T cells in response to specific antigen. We have examined this question with the aid of a well-characterized TCR transgenic model and antigenic peptides with known affinity for TCR and MHC molecules.

Materials and Methods

Mice. 2C TCR transgenic mice were originally obtained from Dr. D. Loh (Nippon Roche Research Center, Kamakura-shi, Japan; reference 18) and were bred and maintained in the rodent breeding colony at The Scripps Research Institute (La Jolla, CA). B10.D2/nSnJ (H-2d) and B10.D2 (R103)/Eg mice (Kd I-Ad I-Ed Db L−) were purchased from The Jackson Laboratory (Bar Harbor, ME).

Media. HBSS supplemented with 2.5% γ-globulin–free horse serum (GIBCO BRL, Santa Clara, CA) was used for preparation

Table 1. *Features of the Interaction of 2C TCR with L^d and Self Peptides*

| Peptides | Sequence | Binding affinities (M^{-1}) (K$_a$) | |
		Affinity for L^d	Affinity of 2C TCR for peptide-L^d
p2Ca	LSPFPFDL	4×10^6	2×10^6
QL9	QLSPFPFDL	2×10^8	2×10^7
SL9	SPFPFDLLL	4×10^7	11.2×10^4
P1A.35-43	LPYLGWLVF	4×10^9	ND

The data on peptide sequences (p2Ca, QL9, and SL9) and the affinity measurements for L^d are taken from Sykulev et al., (26, 48). Affinity measurements for 2C TCR binding to soluble peptide/L^d complexes are based on studies with cell-bound TCR. The affinity of soluble 2C TCR to L^d/p2Ca by BIAcore measurement is 10^{-7} M (50). The sequence of P1A.35-43 peptide is taken from Van den Eynde et al., (27); the affinity of this peptide for L^d was measured by the current authors using methods described previously (26, 48).

of single cell suspensions (19). For proliferation assays, RPMI 1640 was supplemented with 10% fetal calf serum (Irvine Scientific, Santa Ana, CA), 5% NCTC 109, 2 mM glutamine, 5×10^{-5} M 2-ME, and antibiotics.

Antibodies and Reagents. The following mAbs were used for purifying CD8$^+$ 2C or CD8$^-$ 2C cells and were described previously (19): 3.168 (anti-CD8), RL172 (anti-CD4), J11d (anti–heat stable antigen), 28-16-8s (anti–I-A^b). The hybridoma producing 1B2 mAb (clonotypic anti-2C TCR) was provided by Dr. H. Eisen (Massachusetts Institute of Technology, Cambridge, MA). FITC-conjugated 1B2 mAb or biotin-conjugated 1B2 mAb were prepared as previously described. FITC-conjugated anti-CD25, anti-CD69, and PE-conjugated anti-CD8 were purchased from PharMingen (San Diego, CA). Cytochalasin D, cycloheximide, colchicine, and genistein were purchased from Calbiochem Corp. (La Jolla, CA).

Peptides. The peptides used (Table 1) were synthesized on a synthesizer (431 A; Applied Biosystems, Foster City, CA), and were purified with C18 reverse-phase HPLC at the peptide laboratory of R.W. Johnson Pharmaceutical Research Institute (La Jolla, CA). Concentrations of peptides were determined by quantitative amino acid analysis.

Drosophila cell APCs. *Drosophila* APCs were constructed by transfecting Schneider SC2 cells with cDNA for L^d, β2-microglobulin, B7-1, and/or ICAM-1 as described previously (20, 21). The stable cell lines were selected and maintained by culturing the cell lines in the presence of 500 μg/ml geneticin (GIBCO BRL) in culture medium at room temperature (25°C). The genes used for transfection had a metallothionein promoter, and expression of the genes was induced by addition of CuSO$_4$ to a final concentration of 1 mM. For use as APCs, cells cultured with CuSO$_4$ for 24 h were washed three times in HBSS solution containing 2.5% horse serum, and preincubated for 2 h with or without peptides.

Purification of CD8$^+$ and CD8$^-$ 2C Cells. For cell purification (19), 2C LN cells were first treated with a cocktail of mAbs (anti-CD4, anti-HSA, anti–I-A^b) plus C for 45 min at 37°C. The surviving cells were further separated into CD8$^+$ and CD8$^-$ (CD4$^-$) cells by panning at 4°C for 60–90 min on petri dishes coated with anti-CD8 mAb; nearly all of these cells had a naive (CD44^{lo}) phe-

notype (22). Nonattached cells were eluted and treated with anti-CD8 and C to obtain CD8$^-$1B2$^+$ 2C cells. The attached CD8$^+$ 1B2$^+$ 2C cells were recovered by incubation at 37°C for 5 min followed by vigorous pipetting.

Proliferation Assay. Purified populations of CD8$^+$ or CD8$^-$ 2C cells were cultured with irradiated stimulator cells in 200 μl wells (19). B10.D2 spleen cells were exposed to 2,000 cGy, washed, incubated at 5×10^6 cells per ml with or without the indicated peptides at 25°C for 2 h, and then plated out without washing. Responder cells were used at 2.5×10^4 per well and stimulators were used at 5×10^5 per well. To measure T cell proliferation, cultures were pulsed with 1 μCi of [^{3}H]thymidine 8 h before harvest. The data in the figures refer to the mean of triplicate cultures; standard deviations were generally within 5–15% of the mean.

Flow Cytometric Analysis of Surface and Intracellular Molecules. CD8$^+$ or CD8$^-$ 2C cells (0.5×10^6) were cultured with T-depleted spleen cells (5×10^6) or *Drosophila* APCs (1×10^6) in the presence or absence of peptides for the indicated time. The cells were washed once with 3 ml of ice-cold PBS containing 2.5% horse serum and 0.2% sodium azide. For TCR surface staining, cells were incubated with PE-conjugated anti-CD8 mAb and FITC-conjugated 1B2 mAb. For CD25 and CD69 surface staining, cells were incubated with PE-conjugated anti-CD8 mAb plus FITC-conjugated anti-CD25 or FITC-conjugated anti-CD69 mAbs. Propidium iodide was included during staining at a concentration of 1 μg/ml. Live cells (propidium iodide negative) were acquired and analyzed on a FACScan® (Becton Dickinson, San Jose, CA). For surface versus cytoplasmic staining of TCR and CD3, cells were first incubated with red 613–conjugated anti-CD8 plus biotin-conjugated 1B2 mAb or biotin-conjugated anti-CD3 mAb, followed by incubation with PE-conjugated streptavidin. After incubation with unconjugated 1B2 or anti-CD3 mAb (to block unbound sites), the cells were then fixed with 1% paraformaldehyde in PBS containing 0.1% glutaraldehyde and 0.02% Tween 20. The fixed cells were washed and stained with FITC-conjugated 1B2 mAb or anti-CD3 mAb. Cells were analyzed on a FACScan® and the surface and cytoplasmic expression of TCR and CD3 were examined by gating on CD8$^+$ T cells.

Results

Experimental Approach. TCR downregulation on T cells from the 2C line of TCR transgenic mice (18) was examined with the aid of three peptides recognized by the 2C TCR in association with L^d MHC class I molecules. The specificity of 2C cells for L^d-associated peptides is summarized below.

The clonotype-positive (1B2$^+$) CD8$^+$ cells in 2C mice undergo positive selection to K^b (H-2^b) molecules and display strong alloreactivity to endogenous peptides bound to L^d (H-2^d) molecules (18, 23, 24). Based on studies with peptides eluted from cell surface L^d molecules, the alloreactivity of 2C T cells appears to be directed predominantly to an 8-mer peptide, p2Ca, derived from the Krebs cycle enzyme, 2 oxoglutarate dehydrogenase (25); p2Ca peptide has intermediate affinity for soluble L^d molecules and, when complexed to L^d, has high affinity for the 2C TCR (Table 1). 2C T cells display stronger reactivity to a 9-mer variant of p2Ca termed QL9 (26). Except for one additional amino acid, QL9 has the same sequence as p2Ca and forms part of
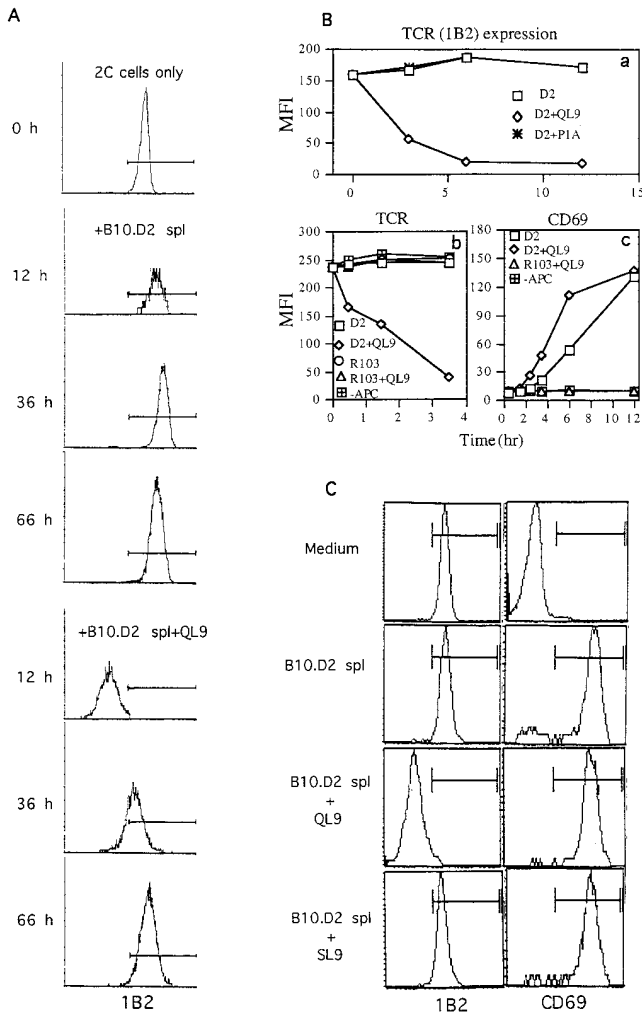
Figure 1. TCR and CD69 expression on CD8^{+} 2C cells stimulated by spleen cells in the absence or presence of peptides. 5×10^5 CD8^{+} 2C cells were cultured with 5×10^6 T-depleted B10.D2 (L[d+]) or R103 (L[d−]) spleen cells in the presence or absence of p2Ca, QL9, SL9, or P1A peptides (10 μM) for the indicated time (12 h for C). The cells were harvested and stained with PE-conjugated anti-CD8 mAb and FITC-conjugated 1B2 mAb. TCR expression was analyzed by FACScan® gating on CD8^{+} 2C cells. In B, the mean fluorescence intensity (*MFI*) of staining is shown. The data in A, B a, B b, B c, and C are from five separate experiments.

the natural sequence of 2 oxoglutarate dehydrogenase. QL9 peptide has very high binding affinity for both L[d] and 2C TCR molecules, i.e., 50-fold (L[d]) and 10-fold (TCR) higher than for p2Ca. The 2C TCR also has specificity for a weaker peptide, SL9 (Table 1). A control peptide, P1A.35-43 (27), has no detectable specificity for the 2C TCR but has high affinity for L[d] molecules (Table 1).

When used in high doses, the three peptides recognized by the 2C TCR are all immunogenic for naive 2C CD8^{+} cells in the absence of added cytokines, and elicit strong proliferative responses, cytokine production, and effector cell generation when presented by L[d]-transfected RMA-S cells as APCs (21); with limiting doses of peptide, the im-

munogenicity of QL9 peptide is about 10,000-fold higher for 2C cells than p2Ca or SL9 peptides. These three peptides are also immunogenic for 2C cells when presented by artificial APCs, i.e., by *Drosophila* cells expressing L[d] molecules and two costimulatory molecules, B7-1 and ICAM-1 (21, and unpublished data of the authors).

In the case of normal spleen cells as APCs, 2C CD8^{+} cells give strong, but brief, proliferative responses and low IL-2 production to B10.D2 (L[d]) spleen cells in the absence of exogenous peptides (19, 22); in this situation, the response is directed to L[d] plus endogenous p2Ca peptide. Supplementing B10.D2 spleen cells (or purified dendritic cells) with exogenous p2Ca or QL9 peptide augments the proliferative response of 2C cells and intensifies IL-2 production, thereby prolonging the proliferative response (22). TCR expression on 2C CD8^{+} cells responding to B10.D2 spleen cells ± exogenous peptides is discussed below.

Spleen Cells as APCs. To examine TCR expression, purified populations of naive phenotype (CD44^{lo}) CD8^{+} cells were prepared from 2C LN and cultured in vitro with L[d]-positive B10.D2 spleen cells or L[d]-negative H-2 recombinant R103 (K[d]D[b]L^{−}) spleen cells ± peptides for up to 3 d. The cells were then harvested, stained for the clonotypic 2C TCR (1B2), for CD69 and CD8 expression, and FACS® analyzed. The data in Fig. 1 show TCR and CD69 expression on gated CD8^{+} cells for cultures harvested after various periods. The data make two points.

First, relative to T cells cultured in medium alone or held at 4°C or cultured with nonstimulating L[d]-negative R103 spleen, no detectable TCR downregulation occurred when 2C cells were cultured with B10.D2 spleen cells in the absence of exogenous peptide (Fig. 1, A, B a, and B b). The failure to detect TCR downregulation applied at all time points measured between 30 min and 66 h of culture (Fig. 1, A and B). Despite the lack of TCR downregulation, contact of 2C cells with endogenous p2Ca peptide on B10.D2 spleen cells caused maximal upregulation of CD69 (Fig. 1, B c and C) and a strong T proliferative response (see below). These data suggest that for 2C cells, TCR downregulation detectable via FACS® analysis is not a prerequisite for T cell activation.

Second, culturing 2C T cells with B10.D2 spleen supplemented with the strong QL9 peptide (10^{-5} M, 10 μM) accelerated the onset of high CD69 expression (relative to B10.D2 spleen without added peptide; Fig. 1 B c), but caused near-complete TCR downregulation (Fig. 1, A, B, and C). TCR downregulation was maximal at 6 h and remained low for 12 h (Fig. 1, A, B a, B b, and data not shown). Thereafter, TCR expression gradually increased (despite continuous exposure to APCs plus peptide) and reached normal levels by 66 h (Fig. 1 A). With the two weaker peptides, p2Ca and SL9, TCR downregulation was undetectable with SL9 (Fig. 1 C) and partial with p2Ca (see below); no TCR downregulation was seen with the control P1A peptide (Fig. 1 B a). These data applied to high concentrations of peptides (10^{-5} M) and with B10.D2 spleen as APCs. In marked contrast to B10.D2 spleen, adding the strong QL9 peptide to nonstimulatory L[d]-negative
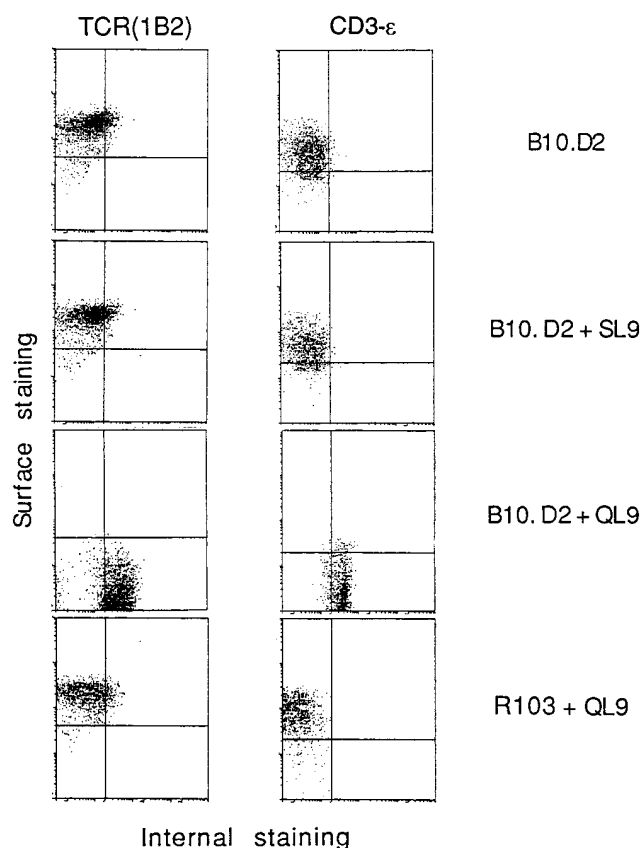
Figure 2. Surface and cytoplasmic staining of TCR and CD3 on CD8+ 2C cells exposed to spleen cells ± peptides. 5×10^5 CD8+ 2C cells were cultured with 5×10^6 B10.D2 or R103 spleen cells in the absence or presence of 10 µM QL9 or SL9 peptides for 12 h. The cells were harvested and stained as described in Materials and Methods. The external and internal expression of TCR and CD3 on the 2C cells were then analyzed by FACScan® gating on CD8+ cells.

R103 spleen failed to cause TCR downregulation or upregulation of CD69 (Fig. 1, *B b* and *B c*).

TCR Downregulation Reflects Internalization. To exclude the possibility that TCR downregulation reflected shedding from the cell surface, 2C T cells were stimulated with B10.D2 spleen ± peptides (10^{-5} M) for 12 h. Using two-color staining, the cells were surface stained for TCR or CD3, permeabilized by fixation, and then stained for TCR and CD3 (CD3ε) internally. As shown in Fig. 2, 2C cells cultured with B10.D2 spleen without added peptide or with SL9 peptide showed high levels of surface (s)[1] TCR and CD3, but only low levels of internal (i) TCR and CD3; similar findings applied when QL9 peptide was added to L^d-negative R103 spleen. With B10.D2 spleen plus QL9 peptide, however, the disappearance of sTCR and sCD3 was associated with a marked increase in iTCR and iCD3. These findings indicate that TCR and CD3 downregulation reflected internalization rather than shedding.

Influence of CD8 Expression on TCR Downregulation. CD8 molecules function by augmenting TCR contact with pep-

tide/MHC class I complexes on APCs and also by promoting intracellular signaling via association with $p56^{lck}$ (28, 29). In the absence of CD8 function, e.g., when anti-CD8 mAb is added to culture, the proliferative response of naive 2C CD8+ cells to B10.D2 spleen without added peptides is undetectable unless the cells are supplemented with exogenous cytokines (19); similar findings apply when the CD8− (CD4−8−) subset of 2C cells are used as responders. With addition of the strong QL9 peptide to B10.D2 spleen cells, however, CD8 function becomes redundant, implying that CD8 function is only required when the avidity of T/APC interaction is low (22). This is illustrated in Fig. 3 *A* where it can be seen that the clonotype-positive (1B2+) subset of CD8− 2C cells failed to proliferate in response to B10.D2 spleen cells without exogenous peptide but gave high responses to B10.D2 spleen supplemented with QL9 peptide (*bottom*). CD8+ 2C cells, by contrast, responded strongly to B10.D2 spleen in the absence of added peptide (*top*). As discussed elsewhere, CD8+ 2C cells behave identically to CD8− 2C cells when supplemented with anti-CD8 mAb (19), implying that the CD8− subset is not innately peculiar.

The above findings indicate that CD8 is an important coreceptor for T cell activation, but is not needed when the avidity of T/APC interaction is high, e.g., when APCs express the high affinity QL9 peptide. This poses the question of whether CD8 expression plays a role in TCR downregulation. This issue was addressed by culturing CD8+ versus CD8− 2C cells with B10.D2 spleen cells supplemented with graded concentrations of QL9 versus p2Ca peptides. As shown in Fig. 3 *B*, the susceptibility of these two subsets of 2C cells to TCR downregulation was indistinguishable. With QL9 peptide, maximal TCR downregulation at 12 h was seen with 10^{-8} M peptide. TCR downregulation failed to impair T cell function because both CD8+ and CD8− 2C cells gave strong proliferative responses to B10.D2 spleen plus QL9 peptide at 10^{-7}–10^{-8} M (Fig. 3 *A*), i.e., at peptide concentrations causing complete TCR downregulation.

For the weaker p2Ca peptide, adding this peptide to either CD8+ or CD8− 2C cells at low concentrations caused a paradoxical mild enhancement of TCR expression (Fig. 3 *B*). At higher concentrations, p2Ca peptide induced TCR downregulation. At the highest concentration tested (10^{-5} M), TCR downregulation by p2Ca peptide was incomplete and equivalent to the partial TCR downregulation induced by a 10,000-fold lower dose of QL9 peptide (10^{-9} M). As discussed earlier (Figs. 1 and 2), the third immunogenic peptide, SL9, failed to cause TCR downregulation at >10^{-5} M.

The above findings indicate that TCR downregulation is (*a*) peptide dose dependent, (*b*) most prominent with high-affinity peptides, (*c*) independent of CD8 expression, and (*d*) apparently unrelated to the subsequent functional response of T cells. On this last point, it may be noted that 2C cells gave strong proliferative responses irrespective of whether TCR expression was upregulated (p2Ca peptide at 10^{-7} M) or completely downregulated (QL9 at 10^{-7} M) (Fig. 3 and data not shown).

Influence of Costimulatory Molecules. Stimulation of T cells

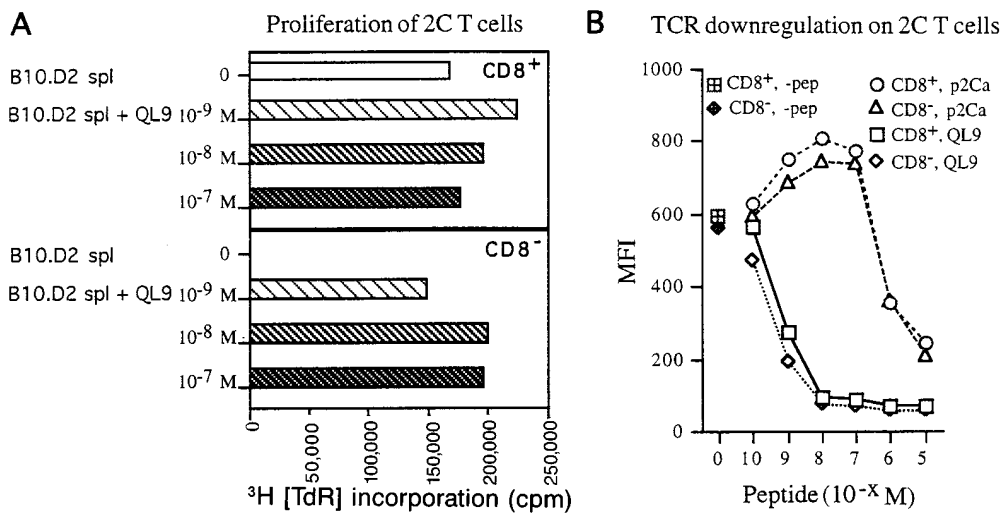[1]*Abbreviations used in this paper:* CCD, cytochalasin D; i, internal; s, surface.

Figure 3. Influence of 2C CD8 expression on TCR downregulation and T cell activation. (A) Proliferative responses of 2C cells to B10.D2 spleen APCs ± QL9 peptide. Purified CD8$^+$ or CD8$^-$ 2C cells (5 × 10^4) were cultured with B10.D2 spleen cells (5 × 10^5) in the absence or presence of titrated concentrations of QL9 peptides for 3 d. [^{3}H]thymidine (1 μCi) was added during the last 8 h of culture. (B) TCR downregulation on 2C T cells. Purified CD8$^+$ or CD8$^-$ 2C T cells (5 × 10^5) were cultured with T-depleted B10.D2 spleen cells (5 × 10^6) in the absence or presence of a titrated concentration of QL9 or p2Ca peptides for 12 h. The cells were harvested and stained for TCR expression with FITC-conjugated 1B2 mAb. The expression of TCR on 2C cells was analyzed on gated Thy-1$^+$ cells.

via TCR molecules is generally ineffective unless accompanied by costimulatory signals (30, 31). These "second signals" are crucial for cytokine production and differentiation into effector cells. Costimulation is thought to be largely a reflection of T cell CD28 molecules interacting with B7 (B7-1, B7-2) on APCs (32, 33). Nevertheless, costimulation can also be provided through interactions between complementary adhesion molecules, e.g., between LFA-1 and ICAM-1 (34–37). Whether this form of costimulation is due solely to enhanced cell adhesion or is also associated with the production of unique second signals is unclear (21, 38). The role of B7 and ICAM-1 in TCR downregulation is discussed below.

In initial experiments, 2C T cells were cultured with B10.D2 spleen cells plus QL9 peptide (10^{-5} M) in the presence of CTLA4Ig, a reagent that binds to B7-1 and B7-2 on APCs and thus blocks CD28/B7 interaction (39). Even at high concentrations, CTLA4Ig had little if any capacity to prevent TCR downregulation (data not shown). Since spleen APCs express a spectrum of molecules with potential costimulatory function, the role of individual costimulatory molecules on TCR downregulation was examined with the aid of a panel of L^d-transfected *Drosophila* cells as APCs. These cells expressed L^d alone (L^d APCs), L^d + B7-1 (L^d.B7), L^d + ICAM-1 (L^d.ICAM), or L^d + B7-1 + ICAM-1 (L^d.B7.ICAM). The capacity of these *Drosophila* APCs to elicit proliferative responses of 2C CD8$^+$ cells in the absence of added cytokines is discussed elsewhere (21) and is summarized in Fig. 4, *right.* For QL9 peptide, proliferative responses are undetectable with L^d APCs, weak but detectable with L^d.B7 or L^d.ICAM APCs, and very strong with L^d.B7.ICAM APCs; for p2Ca peptides, proliferative responses are seen only with L^d.B7.ICAM APCs.

TCR and CD8 expression on CD8$^+$ 2C cells exposed to the panel of *Drosophila* APCs plus QL9 versus p2Ca peptide

for 12 h are shown in Fig. 4; the peptides were added at 10^{-5} M. For p2Ca peptide, TCR expression was high or only slightly reduced with each type of *Drosophila* APC. With QL9 peptide, by contrast, TCR expression was greatly reduced, even with *Drosophila* cells expressing L^d alone. Therefore, TCR downregulation did not appear to require costimulation. Similar findings applied to CD3 expression (data not shown), implying that TCR downregulation did not simply reflect TCR blockade. In contrast to TCR and CD3 expression, neither peptide caused a change in CD8
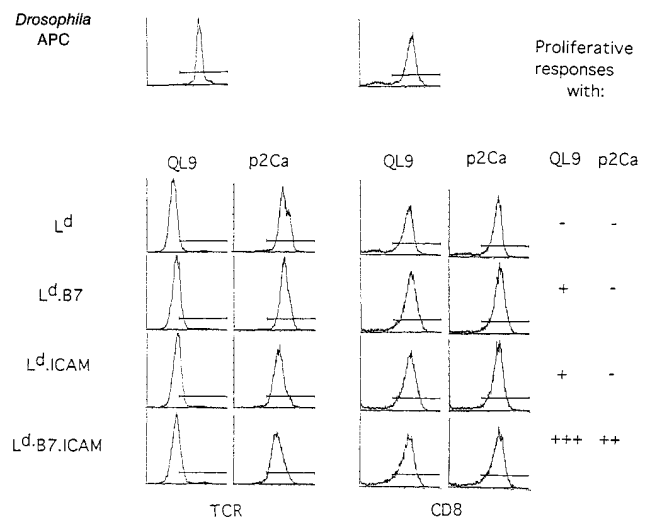

Figure 4. Influence of costimulation on TCR downregulation and T cell activation. Purified CD8$^+$ 2C cells (5 × 10^5) were incubated with the indicated transfected *Drosophila* cells (1 × 10^6) plus p2Ca or QL9 peptides (10 μM) in bulk (2 ml) culture for 12 h and stained for TCR (1B2) and CD8 expression. (*Top*) staining of noncultured 2C cells is shown as a control. The summarized data on proliferative responses of 2C cells to p2Ca and QL9 peptides are taken from Cai et al. (21).
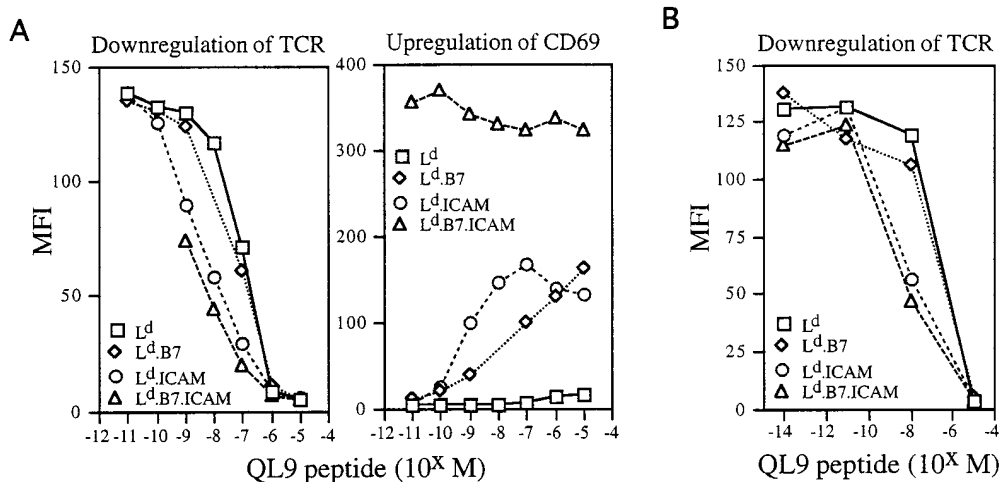
Figure 5. Influence of B7-1 and ICAM-1 expression on peptide-induced 2C TCR downregulation. (*A*) 5×10^5 purified $CD8^+$ 2C cells were cultured with 1×10^6 *Drosophila* APCs in the presence of a titrated concentration of QL9 peptides for 12 h. The cells were harvested and stained with FITC-conjugated 1B2 or FITC-conjugated anti-CD69. The data show TCR and CD69 expression on gated $CD8^+$ 2C cells. The data in *A* and *B* are from two separate experiments.

expression (Fig. 4). These findings with naive 2C cells also applied to presensitized T cells, i.e., to 2C cells harvested 3 d after exposure to L^d.B7.ICAM APCs plus QL9 peptide (data not shown).

The above data apply to high concentrations of peptides (10^{-5} M). The effects of using limiting doses of QL9 peptide are shown in Fig. 5. Under these conditions, the capacity of L^d and L^d.B7 APCs to cause QL9-mediated TCR downregulation of naive 2C cells was virtually identical, implying that B7-1 expression played no detectable role in TCR
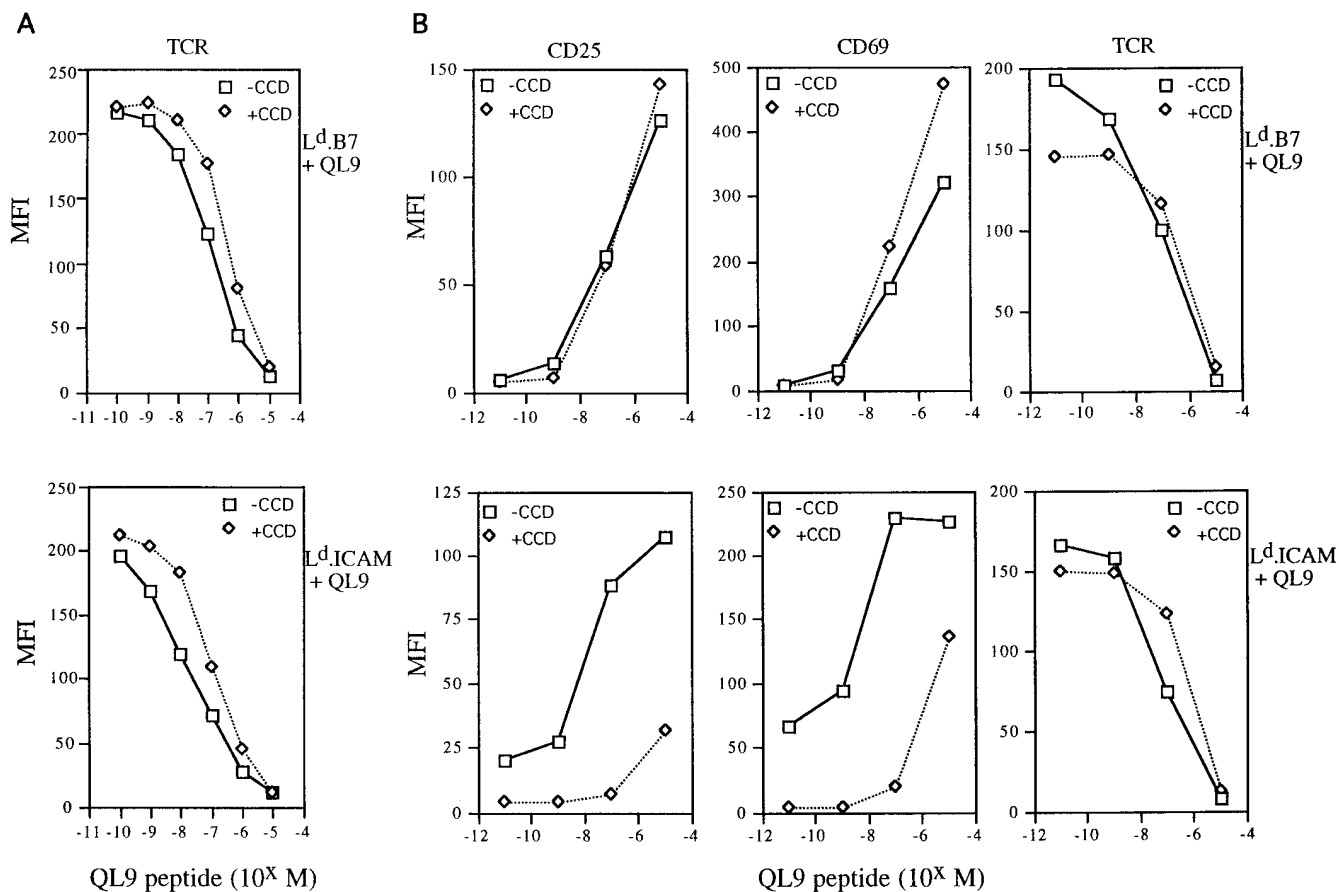

Figure 6. Effects of CCD on TCR downregulation and T cell activation. Purified $CD8^+$ 2C cells (5×10^5) were cultured with 1×10^6 *Drosophila* APCs (*top*, L^d.B7 cells and *bottom* L^d.ICAM cells) in the presence of a titrated dose of QL9 peptide for 12 h. CCD was added at the beginning of the culture at a final concentration of 4 μg/ml. The cells were harvested and stained for FITC-conjugated anti-CD25, FITC-conjugated anti-CD69, or FITC-conjugated 1B2 (TCR) mAbs. The cells were analyzed on a FACScan and the data show the mean fluorescence intensity (*MFI*) on gated $CD8^+$ 2C cells stained with each mAb. The data in *A* and *B* are from different experiments.

downregulation. Interestingly, in contrast to B7-1, ICAM-1 expression appeared to potentiate TCR downregulation. Thus, at limiting doses of peptides (10^{-7}–10^{-9} M) TCR downregulation was more pronounced with ICAM-1$^+$ APCs (L^d.ICAM or L^d.B7.ICAM cells) than with ICAM-1$^-$ APCs (L^d or L^d.B7 APCs; Fig. 5 *A*, *left*). Several other experiments, e.g., Fig. 5 *B*, gave similar results.

These findings with limiting concentrations of QL9 peptide indicated that TCR downregulation did not require typical costimulation via B7-1, but was significantly augmented by contact with ICAM-1. Quite different results occurred when the T cells were typed for CD69 expression. Thus, in the presence of graded concentrations of QL9 peptide, upregulation of CD69 expression on 2C cells was virtually undetectable with L^d APCs, moderate with either L^d.B7 or L^d.ICAM APCs, and very high with L^d.B7.ICAM APC (Fig. 5 *A*, *right*). The requirements for inducing TCR downregulation and T cell activation (CD69 upregulation) thus seemed to be unrelated. Except for a mild effect of ICAM-1 at low peptide concentrations, TCR downregulation appeared to depend solely on contact with L^d molecules. By contrast, T cell activation was critically dependent upon costimulation provided either by B7-1 or ICAM-1; optimal T cell stimulation required the combined expression of B7-1 and ICAM-1.

Since TCR downregulation was enhanced only by ICAM-1 and not B7-1, it seemed likely that ICAM-1 acted simply by promoting conjugate formation (cell adhesion), i.e., by binding to LFA-1 coreceptors on 2C cells and thus facilitating TCR contact with L^d + peptide on the APCs. However, the possibility that ICAM-1 initiated unique signaling events that potentiated TCR downregulation could not be excluded. Since LFA-1 interaction with ICAM-1 involves LFA-1 association with the cytoskeleton (40), cultures were supplemented with cytochalasin D (CCD), a drug that inhibits actin filament polymerization (41).

The effects of adding CCD to 2C cells cultured with L^d.B7 versus L^d.ICAM *Drosophila* APCs plus graded concentrations of QL9 peptide are shown in Fig. 6 *A*. In the case of TCR expression, CCD had only a very mild effect in reducing TCR downregulation. This effect was seen in several different experiments and tended to be slightly more pronounced for L^d.ICAM than L^d.B7 APCs (see also Fig. 6 *B*, *right*). For CD25 (IL-2Rα) and CD69 expression, however, the results were much more dramatic (Fig. 6 *B*). Thus, with L^d.ICAM APCs, addition of CCD profoundly reduced upregulation of CD25 and CD69 expression, especially at low concentrations of peptide. With L^d.B7 APCs, by contrast, CCD failed to impair CD25 or CD69 upregulation; indeed, the upregulation of these markers was slightly increased in the presence of CCD. The implications of these findings will be discussed later.

Metabolic Requirements for TCR Downregulation. The capacity of various metabolic inhibitors to impair TCR downregulation was measured with B10.D2 spleen cells as APCs ± QL9 peptide (10^{-5} M); drugs were dissolved in DMSO and TCR expression on CD8$^+$ 2C cells was examined at 12 h. As shown in Fig. 7, relatively high concentra-
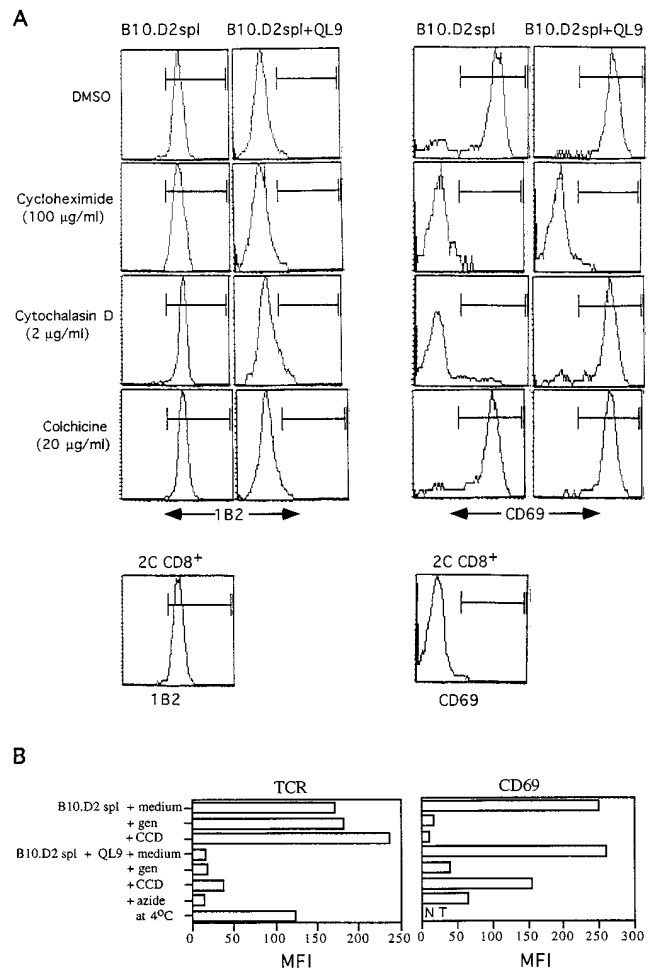
Figure 7. Metabolic requirements for TCR downregulation. CD8$^+$ 2C cells (1×10^6) were cultured with 5×10^6 CD8-depleted B10.D2 spleen cells in the presence or absence of 10 μM QL9 peptides for 12 h. Inhibitors were added at the beginning of the cultures as indicated. Cells were harvested and stained for the expression of TCR (1B2) and CD69 as described in Materials and Methods. Staining for TCR and CD69 expression is shown in the form of histograms (*A*) and MFI (*B*) and was obtained by gating on CD8$^+$ cells. In *A*, the histograms at the bottom refer to staining of control 2C cells cultured at 4°C in the absence of APCs. In *B*, the inhibitors were added at 200 μM for genistein, 4 μg/ml for CCD, and 0.2% for sodium azide.

tions of cycloheximide (100 μg/ml, inhibitor of protein synthesis), colchicine (20 μg/ml, inhibitor of microtubule polymerization; 42), CCD (see above), genistein (200 μM, inhibitor of tyrosine-specific protein kinase; 43) or sodium azide (0.2%, cytochrome oxidase inhibitor) had no detectable effect in inhibiting TCR downregulation at the time point studied. However, with the exception of colchicine, each drug prevented CD69 upregulation (though in accordance with the data in Fig. 6, the inhibition induced by CCD was prominent only when the B10.D2 APCs were not supplemented with QL9 peptide). Higher concentrations of drugs, e.g., 1% sodium azide, did reduce TCR downregulation, but substantially impaired cell viability, implying that the effects were nonspecific. The only approach tested that prevented TCR downregulation without causing cell damage was to culture cells at 4°C (Fig. 7 *B*).

Discussion

Under physiological conditions, TCR binding to peptide/MHC complexes on APCs elicits T cell activation via an intracellular signaling cascade involving a series of phosphorylation-driven events (44). However, TCR-mediated signaling is generally abortive unless amplified by co-recognition of MHC molecules by CD4 or CD8 molecules and accompanied by the delivery of "second signals" through recognition of costimulatory molecules, e.g., B7, on APCs (2, 30). The precise requirements for inducing signal transduction via TCR/CD3 molecules is unclear (44). The prevailing view is that signaling reflects TCR/CD3 cross-linking which leads to conformational changes in these molecules and activation of intracellular protein tyrosine kinases such as $p56^{lck}$ and fyn. As mentioned earlier (see Introduction), an alternative possibility is that T cell activation is not initiated by TCR/CD3 cross-linking per se, but by internalization of these components (17). A key question here is whether such TCR downregulation is an essential prelude to signaling or merely an epiphenomenon.

In this paper we compared the requirements for inducing TCR downregulation versus T cell activation. This question was addressed with the aid of a well-characterized system in which a monoclonal population of naive T cells was exposed to specific peptides presented by APCs bearing defined costimulatory molecules. The results show that the requirements for inducing TCR downregulation and T cell activation show little or no correlation. Confirming previous findings (21), the activation of 2C T cells leading to CD69 upregulation, CD25 expression, and cell division required costimulation via B7 or ICAM-1. Except with the high affinity QL9 peptide, 2C activation was also dependent upon the coreceptor function of CD8. These requirements did not apply to TCR downregulation. Thus, with QL9 peptide, induction of TCR downregulation on 2C cells appeared to depend simply upon peptide/MHC interaction and was not influenced by either CD8 or B7 expression. With regard to B7, in other experiments we observed QL9-mediated TCR downregulation of 2C cells with a number of $B7^-$ $ICAM-1^{-/lo}$ cell lines (P815 cells, L^d-transfected fibroblasts, L^d-transfected RMA-S cells) and also with 5μ Dynabeads coupled with $QL9/L^d$ complexes (authors and A. Luxemberg, unpublished data). Induction of TCR downregulation in the absence of costimulation was thus not unique to Ld *Drosophila* cells.

TCR downregulation was clearly not sufficient for T cell activation because exposure to QL9 peptide on *Drosophila* cells expressing L^d alone caused marked TCR downregulation, but only minimal signs of T cell activation (limited to a slight increase in CD69 expression with high doses of peptide). Conversely, TCR downregulation did not seem to be mandatory for T cell activation because culturing 2C cells with B10.D2 spleen without exogenous peptide stimulated a strong proliferative response but failed to cause detectable TCR downregulation. This finding should be viewed with caution, however, because FACS® analysis may not be sufficient to detect a minor degree of TCR

downregulation. Thus, Viola and Lanzavecchia (16) argue that T cell activation requires downregulation of only a small proportion of total TCR molecules, i.e., ~8,000 molecules per cell with $B7^-$ APCs and 1,500 molecules per cell with $B7^+$ APCs. Although it is difficult to definitively rule out this level of TCR downregulation, it is notable that adding limiting doses of the weaker p2Ca peptide to B10.D2 spleen potentiated the proliferative response (22), but caused a paradoxical increase rather that a decrease in TCR expression (Fig. 3). These findings are difficult to reconcile with the notion that TCR downregulation is a prerequisite for T cell activation. Nevertheless definitive information on this question will require the use of more sensitive methods for quantitating TCR levels. At present, the possibility that the cells downregulated a very small but significant number of TCR molecules cannot be excluded.

Although TCR downregulation was not influenced by B7 expression, it is of interest that, with *Drosophila* cells as APCs, TCR downregulation was slightly enhanced by L^d.ICAM cells relative to either L^d or L^d.B7 cells. The simplest explanation for these data is that TCR downregulation does not require classic costimulation but is facilitated by the enhanced conjugate formation induced by the adhesive function of LFA-1/ICAM-1 interaction. It is important to stress, however, that the role of ICAM-1 in augmenting TCR downregulation was quite mild and was only seen with limiting doses of peptides. This contrasted with the requirements for T cell activation where ICAM-1 had a decisive influence. Thus, induction of CD69 expression was barely detectable with L^d APCs, but was quite strong with L^d.ICAM APCs, in fact, as strong as with L^d.B7 APCs. ICAM-1 thus seemed to play only a minor role in TCR downregulation, but had a major influence on T cell activation. This difference implies that the role of LFA-1/ICAM-1 interactions in TCR downregulation and T cell activation is distinctly different. How can this be explained?

Although the interaction between LFA-1 and ICAM-1 is known to augment cell adhesion (45), this interaction is also reported to provide costimulatory or "coactivation" function (34–37), implying that LFA-1/ICAM-1 interaction can be bifunctional. During the initial interaction between T cells and APCs, LFA-1/ICAM-1 interaction probably acts primarily by enhancing cell adhesion, thus facilitating contact with APCs expressing limiting concentrations of peptide. The present results with CCD could be viewed as supporting the concept that the adhesive function of LFA-1 is not constitutive on resting T cells (34, 45), but requires association with the cytoskeleton, presumably as the result of earlier TCR ligation. This may be an overinterpretation of the data, however, because the effects of CCD in preventing TCR downregulation were minor and were only marginally greater for L^d.ICAM than for L^d.B7 APCs.

After initial T/APC interaction, one can envisage that the adhesive function of LFA-1/ICAM-1 interaction continues, but that LFA-1 now assumes a role as a costimulatory molecule. As documented here and elsewhere (21),

ICAM-1 and B7 each act as quite potent costimulatory molecules when expressed on *Drosophila* APCs. It is notable, however, that CCD markedly impaired the costimulatory function of ICAM-1, but had little effect on the role of B7. This finding raises the possibility that ICAM-1 and B7 use different signaling pathways for costimulation, which could explain the marked synergism observed when these two molecules are coexpressed on APCs (21, 38).

Though minor, the role of LFA-1/ICAM-1 interaction in TCR downregulation was clearly detectable. This contrasted with CD8 expression where TCR downregulation on 2C cells was as marked with CD8$^-$ cells as with CD8$^+$ cells over a wide range of peptide concentrations. The apparent CD8 independence of TCR downregulation might seem surprising since CD8 interaction with class I is thought to play a significant role in augmenting TCR/peptide/class I interaction (29, 46). For 2C cells, CD8 expression is clearly important for T cell activation to p2Ca and related peptides, especially with limiting concentrations of peptide (19, 22, 47, Fig. 3). This may not be the case for initial TCR/peptide/class I interaction, however, because the binding of soluble p2Ca/L^d complexes to intact CD8$^+$ 2C cells cannot be inhibited with anti-CD8 mAb (48). Therefore, the implication is that for the interaction of cell-bound 2C TCR molecules with the high affinity p2Ca and QL9 peptides, CD8 acts solely as a triggering molecule, presumably by focusing p56^{lck} in the vicinity of the TCR/CD3 complex (26). This function is crucial for cell activation, but not for TCR downregulation.

Collectively, the data on ICAM-1, B7, and CD8 expression suggest that these molecules play a crucial role in T cell triggering, but only a minor or undetectable role in TCR downregulation. What, then, are the requirements for inducing TCR downregulation? According to others, TCR downregulation is an active process requiring tyrosine and/or serine phosphorylation of intracellular proteins after activation of protein kinase C (for review see reference 11). However, these studies involved exposing T cells to phorbol esters and/or the use of T cell clones, and did not exclude secondary effects mediated by contaminating APCs. Hence it is unclear whether protein phosphorylation is essential for TCR downregulation at the level of naive T cells. In this respect, it is notable that the tyrosine kinase inhibitor, genistein, was fully effective in blocking 2C T cell activation (CD69 upregulation), but failed to impair TCR downregulation even at relatively high concentrations (200 μM) (Fig. 7). Several other metabolic inhibitors including sodium azide and cycloheximide had similar effects. It is therefore conceivable that TCR downregulation simply reflects a conformational change in TCR (and/or CD3) components which targets these molecules for endocytosis via clathrin-coated pits. Whatever the explanation, TCR downregulation seems to be neither sufficient nor essential for cell activation. We favor the view that TCR downregulation is a byproduct of strong TCR ligation. Since TCR downregulation is most prominent with high affinity peptides, internalization of the TCR could be a protective measure to guard against the negative effects of excessive T cell triggering. In favor of this idea, recent evidence of Valitutti et al. suggests that TCR downregulation can lead to "extinction of signaling" (49).

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